CHAPTER I

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INTRODUCTION

Although aconitase has been known for some sixteen years, it has not been isolated in the pure state. Ochoa (1948) was able to concentrate the enzyme by ammonium sulphate fractionation, but there was no increase in the specific activity and no further attempts were made to purify it. Buchanan & Anfinsen (1949) obtained a 23-fold purification of aconitase by low temperature ethanol and ammonium sulphate fractionation. Electrophoretic analysis showed that the preparation consisted of "three non-homogeneous components" and the purity of the enzyme was estimated to be 30%.

The purification of aconitase has been greatly hampered by its apparent instability. Krebs & Eggleston (1944) found that glycerol stabilized crude enzyme extracts, but Buchanan & Anfinsen (1949) reported that
glycerol was without effect in stabilizing purified preparations. They also found that whilst cysteine stabilized the crude enzyme, it strongly inhibited the purified enzyme. Citric or cis-aconitic acid were found to be the most effective stabilizers of aconitase.

An important finding for the further study of the purification of aconitase was made by Dickman & Cloutier (1950). They found that crude solutions of aconitase could be both stabilized and activated by the addition of \( Fe^{2+} \) and cysteine. Further work (Dickman & Cloutier, 1951) showed that these agents were also effective in stabilizing and activating more highly purified solutions of the enzyme. \( Fe^{2+} \) was the only cation which gave consistent activation of aconitase. Ascorbic acid was as effective as cysteine as a reducing agent, whilst glutathione was only one half as effective.

An attempt was made to purify the enzyme further in the light of the findings of Dickman & Cloutier (1950, 1951).

**EXPERIMENTAL**

**Citric acid estimation**

The method of Hucher, Sherman & Vickory (1936) as modified by Buffa & Peters (1949) was used. Samples
ranging in volume from 1.0 to 3.0 ml. and containing up to 200 μg. of citric acid were used; within this range the colour given by the method was proportional to the amount of citric acid present.

**Protein estimation**

The protein concentration of aconitase solutions was determined according to Kalckar (1947) by measurement of the absorption in the Beckman spectrophotometer at a wavelength of 280 μm after the appropriate dilution with 0.1 N NaOH. Under these conditions, a solution containing 1 mg. of protein/ml. was found to have an absorption of 1.5.

**Activation of aconitase**

A suitable sample of the enzyme solution was measured into a test tube and placed in an ice-bath. Non-neutralised ferrous ammonium sulphate and neutralised cysteine were added, so that on dilution of the solution with cold water to 10 ml., the final concentration of Fe$^{2+}$ was $5 \times 10^{-4}$ M and that of cysteine was $10^{-2}$ M. The mixture was then neutralised with 8 NaOH to pH 7.4 using a glass electrode and incubated in the ice-bath for 1 hr. before the aconitase activity was determined.

Except where otherwise stated, the various enzyme
fractions were treated in this fashion before an activity determination was carried out.

Aconitase activity determination

To a series of test tubes 4.4 or 4.3 ml. of water were added. 0.5 ml. of a solution containing 20 μmoles of cis-aconitic acid at pH 7.4 was then added and the test tubes placed in a water-bath at 30°. After an equilibration period of 5 min., the reaction was started by the addition of 0.1 or 0.2 ml. of enzyme solution. The reaction was stopped after 15 min. by the addition of 0.5 ml. of 50% (w/v) trichloroacetic acid. If a visible precipitate formed it was filtered off through a No. 30 Whatman filter paper, otherwise the citric acid estimation was carried out on the acidified solution without further treatment. It was found that small amounts of protein did not interfere with the estimation. Under these conditions the amount of citric acid formed from cis-aconitic acid was proportional to the amount of enzyme added. The activity determinations were carried out at two enzyme levels to ensure that the substrate concentration was not limiting.

Definition of a unit of aconitase activity

One unit of aconitase activity was taken to be the
amount of enzyme which formed one μmole of citric acid from cis-aconitic acid in 15 min. at pH 7.4 and 30° in the absence of buffer.

Specific activity

Units of aconitase activity/mg. of protein was taken to be the specific activity.

Other enzyme activity determinations

isocitric dehydrogenase activity was determined by the method of Schoen (1951). One unit was defined as a change of log. 10/1 of 0.01/min. Lumarase activity was determined by the method of Kacker (1950). One unit was defined as a change of log. 10/1 of 0.001/min.

Reagents

cis-Aconitic anhydride (m.p. 77°) was prepared from trans-aconitic acid by the method of Malachowski & Malchow (1928). Sodium cis-aconitate was formed by neutralizing the anhydride to pH 7.4 with N NaOH. Trichloromethyl paraconic acid was prepared by the method of Sittig & Miller (1889) and converted to DL-isocitric lactone by the method of Arbeas & Eggleston (1944). Sodium isocitrate was formed by hydrolysis of the lactone with NaOH as described by Arbeas & Eggleston (1944). I am grateful to Mr. R.W. Nekelin for preparing these two acids.
Absolute ethanol obtained commercially was used throughout. L-Cysteine hydrochloride used in the later work was a Roche product which was free from iron. The ammonium sulphate was B.D.H. Special Grade and the 2:2'-dipyridyl was a B.D.H. Spot Test Reagent. All other reagents were A.R.

RESULTS

Activation of aconitase

The work of Dickman & Cloutier (1951) on the activation of aconitase by Fe$^{2+}$ and cysteine was repeated using their partially purified enzyme preparation. The results were in good agreement with those obtained by those authors. However, the activating effect of cysteine is open to criticism. It was found that the preparation of cysteine which gave similar results to those of Dickman & Cloutier contained appreciable amounts of iron. Nevertheless, the importance of Fe$^{2+}$ and cysteine as activators of aconitase was confirmed. The cysteine used in the later experiments was completely free from iron.

Inactivation of aconitase

It was apparent that the presence of ions, such as phosphate, which are capable of forming insoluble complexes
with \( Fe^{2+} \). would lead to inactivation of the system as a whole. In order to test this experimentally, samples of the same enzyme preparation were activated and pre-incubated at 30°C with 0.05 M phosphate buffer (pH 7.4) for various periods of time. Substrate was then added and the reaction allowed to proceed. In the control system, water replaced the buffer. Fig. 1 shows that phosphate causes rapid inactivation of the activated enzyme when substrate is absent. When phosphate and substrate are added simultaneously, the enzyme activity is greater than that obtained in the absence of phosphate. This increased enzyme activity was shown to be due to the influence of the ions present in solution on the pH optimum of the enzyme (see chapter III). The enzyme activity was not increased by the simultaneous addition of excess \( Fe^{2+} (5 \times 10^{-3} M) \) and substrate, following pre-incubation for various periods of time in the presence of phosphate. The above finding is in contrast to the results of Dickman & Cloutier (1951) for they found that phosphate did not decrease the activity of an activated enzyme preparation. As similar results were obtained with veronal acetate, borate and glycerophosphate buffers at pH 7.4, buffers were not used during the course of enzyme
**Fig. 1.** The effect of incubating aconitase in the absence of substrate. The enzyme was activated with Fe$^{2+}$ and cysteine and incubated at $30^\circ$ for various periods of time. cis-Aconitic acid was added (final concentration $4 \times 10^{-3}$ M) and the reaction was stopped after 15 min. by the addition of 0.5 ml. of 50% trichloroacetic acid. The solutions were analysed for citric acid.

- O No buffer present, reagents adjusted to pH 7.4.
- O.05 M phosphate buffer (pH 7.4)
purification nor for the determination of enzyme activity. A check of the pH of the system before and after incubation showed that although there was a small alteration of the pH in the absence of buffer, the results were reproducible.

Preparation of aconitase by the method of Buchanan & Anfinsen

As Fe$^{2+}$ and cysteine had not been used by Buchanan & Anfinsen (1949) for activation of their aconitase fractions, it was possible that their final preparation was, in fact, of higher specific activity than that reported. In order to determine whether or not this was the case, aconitase was prepared according to the method of these workers. A study was then made of the activity of various fractions in the presence and absence of Fe$^{2+}$ and cysteine. Buchanan & Anfinsen carried out their tests at 38$^\circ$; in the present work the tests were made at 30$^\circ$ and the activities were multiplied by 1.5, the temperature coefficient of the reaction found for 38$^\circ$/30$^\circ$.

The results of table 1 clearly show that in the absence of Fe$^{2+}$ and cysteine, the specific activities of the intermediate fractions are much lower than those claimed by Buchanan & Anfinsen. It was only in the presence of these two agents that similar activities
TABLE 1. Comparison of Aconitase Activities in the Presence and Absence of Fe$^{2+}$ and Cysteine

(Fractions were prepared according to the method of Buchanan & Anfinsen (1949). Activation by Fe$^{2+}$ and cysteine was carried out as described in the text.

Temp. 30°C, pH 7.4. The results obtained were multiplied by 1.5, the temperature coefficient for 38°C/30°C, so that they are equivalent to activities at 38°C).

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Buchanan &amp; Anfinsen results</th>
<th>Present results</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No additions</td>
<td>No additions</td>
</tr>
<tr>
<td></td>
<td>Total units activity</td>
<td>Total units activity</td>
</tr>
<tr>
<td>Extract</td>
<td>310,000 10.0</td>
<td>261,000 10.5</td>
</tr>
<tr>
<td>pH 5.6 ethanol ppt.</td>
<td>133,000 18.9</td>
<td>144,000 26.9</td>
</tr>
<tr>
<td>pH 5.6 ethanol ppt.</td>
<td>102,000 68</td>
<td>100,500 29.3</td>
</tr>
<tr>
<td>pH 6.8 ethanol ppt.</td>
<td>63,000 132</td>
<td>45,000 45</td>
</tr>
</tbody>
</table>
could be obtained. A comparison of the activities of the activated and non-activated fractions shows that the degree of activation by \( \text{Fe}^{2+} \) and cysteine increases as purification proceeds, indicating that the co-factors of the enzyme are removed to some extent by the fractionation procedure. It will also be noted that the relative purification of the enzyme in each step differs markedly from that obtained by Buchanan & Anfinsen. Moreover, it was found that the addition of ammonium sulphate to 66% saturation did not increase the specific activity of the enzyme.

As the method of Buchanan & Anfinsen (1949) gave only a relatively small increase in the purity of acositase, an effort was made to obtain a more highly purified preparation of the enzyme. The procedure utilised by these authors was developed further and some modifications of the initial fractionation steps were made. In view of the fact that maximum enzyme activity was obtained only after the addition of \( \text{Fe}^{2+} \) and cysteine, the various fractions were incubated with these agents before the activities were determined.
METHOD OF ISOLATION OF ACONITASE FROM PIG HEART

It will be appreciated that the attempts to purify aconitase further involved the trial of many purification procedures. The results of all these procedures are not described in detail as many brought about little or no increase in the purity of the enzyme, but brief mention of their use is made. The final method adopted for the enzyme purification was as follows:

Extraction of tissue

Immediately after the death of the animal, the hearts were removed, cut into strips of about 1 inch thickness, freed from fat and connective tissue and placed in ice. At the laboratory, the hearts were minced through a coarse mincer and stored at \(-15^\circ\). Under these conditions, pig heart could be stored for some months without loss of enzyme activity. One kg. of frozen heart was allowed to thaw at room temperature and then homogenised in 200 g. lots for 2 min. in a pre-cooled Waring blender with 500 ml. of 0.004 M citrate buffer (pH 4.7) and 130 ml. of chloroform. Chloroform reduced the haemoglobin content of the extract without denaturation of the enzyme and increased its specific activity. The homogenate was centrifuged at \(-1^\circ\) for
15 min. at 1,000 g. The clear red-amber supernatant (pH 5.7) was poured off and filtered through a fluted filter paper (Whatman No. 53). Below pH 5.0 marked loss of enzyme activity occurred.

**1st. ethanol fractionation**

For convenience, the supernatant was divided into two equal amounts and fractionated simultaneously. From this stage onwards, all operations were carried out in the coldroom. The supernatant was brought to 0° in a dry ice-ethanol bath and 90% ethanol was added with mechanical stirring at the rate of 250 to 300 ml/hr., to a concentration of 15%. (90% ethanol was used throughout the fractionation). Freezing of the solution at this stage leads to loss of enzyme activity. Hyflo Super-Cel was added to the solution (5 g./l.) and filtration was carried out on a Buchner funnel under slight pressure (Whatman No. 30 filter paper). To the clear red-amber filtrate, saturated sodium chloride (1.25 ml./100 ml. of filtrate) was added. The ethanol concentration of the solution was brought to 45%, the temperature being lowered to -10°. The mixture was allowed to stand overnight as contact of the enzyme with ethanol for this period did not have any harmful effect.
2nd. ethanol fractionation

The precipitate was centrifuged off at $-10^\circ$ and stored at the same temperature whilst another kg. of pig heart was treated in the same fashion. The two precipitates were combined and dissolved in 800 ml. of ice-cold water. A small amount of insoluble matter was centrifuged off to yield a clear deep red-amber solution. The solution was brought to $0^\circ$ and ethanol added to a concentration of 10% (the ethanol already present was neglected), the temperature being lowered to $-4^\circ$. A small amount of a white precipitate was centrifuged off at $-4^\circ$. The pH of the supernatant which was about 5.6, was adjusted to pH 6.6 with 5% sodium carbonate solution. Ethanol was added to a concentration of 23% whilst the temperature was lowered to $-10^\circ$. The precipitate was centrifuged off at $-10^\circ$ and dissolved in 100 ml. of ice-cold water.

Ammonium sulphate fractionation

The aqueous solution was diluted 2.5 times with 0.125 M citrate buffer (pH 6.6). Ammonium sulphate (36.7 g./100 ml. of solution) was added slowly, the temperature being lowered to $-6^\circ$. The yellow coloured precipitate was centrifuged off at $-6^\circ$ for 10 min. at 5,000 g. To the supernatant, ammonium sulphate
(9.2 g/100 ml. of supernatant) was added slowly and the temperature lowered to -10°. The precipitate was centrifuged off at -10° for 15 min. at 5,000 g. and dissolved in 50 ml. of 0.064 M citrate buffer (pH 5.7) to give a deep amber solution. This solution was dialysed overnight with stirring against the same buffer. There was no loss of enzyme activity, but dialysis against water and salt solutions caused large losses of enzyme activity.

**Heat fractionation**

The dialysed solution in 20 to 25 ml. lots was heated in a water-bath for 15 min. at 50° and cooled rapidly in an ice-bath. The white flocculent precipitate was centrifuged off at 0° and discarded.

**Ethanol fractionation at pH 8.0.**

The supernatant was cooled to 0° and ethanol was added to a concentration of 20%, the temperature being lowered to -5°. A small amount of precipitate was centrifuged off at -5° and discarded. The supernatant was adjusted to pH 8.0 with a saturated solution of sodium bicarbonate and ethanol was added to a concentration of 50%, the temperature being lowered to -12°. During the fractionation at alkaline pH values, care was taken to prevent CO₂ from distilling over from the dry
ice-ethanol bath into the protein solution. The reddish
coloured precipitate was centrifuged off from a cloudy
supernatant (due to the presence of sodium bicarbonate)
at \(-12^0\) and dissolved in 20 ml. of 0.004 M citrate buffer
(pH 5.7).

Ammonium sulphate fractionation at pH 8.5

A saturated solution of ammonium sulphate was
neutralised with strong ammonia, so that on dilution of
1 in 5, the pH was 8.5. After adjustment of the pH of
the protein solution with ammonia, the ammonium sulphate
solution was added dropwise until the saturation was 0.65.
The temperature was gradually lowered to \(-10^0\). The
precipitate was centrifuged off at \(-10^0\), dissolved in
10 ml. of 0.004 M citrate buffer (pH 5.7) and dialysed
against the same buffer overnight at 0\(^0\). The final
solution was amber in colour.

Attempts to purify the enzyme further were
unsuccessful. Although the enzyme constituted the
major portion of the final solution, it could not be
induced to crystallize. The slow addition of ethanol
always gave rise to an amorphous product. Attempts
were also made to crystallize the enzyme from ammonium
sulphate solutions by the addition of an amount just
insufficient to cause precipitation, followed by adjustment of the pH. Within the pH range 5.7 to 8.5, both in the presence and absence of Fe$^{2+}$ and cysteine, the enzyme precipitated only in small amounts and in an amorphous form.

A brief account of the procedures which were tried without success during the course of the purification follows below. Large amounts of calcium phosphate gel were required to adsorb the enzyme from solution between pH 5.6 and 6.6 and no differential adsorption or elution could be obtained. Although the enzyme could be stirred with n-butanol for 30 min. at room temperature, this treatment did not alter the precipitation pattern after further ethanol fractionation. No protein was precipitated by the nucleic precipitants manganese sulphate and protamine sulphate. Fractionation with acetone precipitated the enzyme between 30 and 50% acetone concentration, but marked loss of activity occurred even though the fractionation was carried out between -5$^\circ$ and -10$^\circ$. This finding is in contrast to that of Buchanan & Anfinsen (1949), but is consistent with the fact that acetone powders of pig heart are devoid of aconitase activity. The enzyme was not precipitated by sodium
chloride or ammonium acetate. Alcohol fractionation in the presence of zinc was ineffective, presumably because the zinc reacted with the citrate present.

In Table 2 is given a summary of the yields and degrees of purification of aconitase during the fractionation procedure. This procedure brings about a 24-fold increase in the purity of aconitase, with an overall recovery of about 12%. The final product has a specific activity of about 265 when the test is carried out in the absence of buffer. If the test is carried out in 0.05 M phosphate buffer (pH 7.4), the specific activity is increased to 336. As mentioned previously, this increase is due to the fact that the pH optimum of aconitase is dependent upon the ions present in solution (see Chapter III). It was calculated that at 38° the specific activity is about 500 which compares to the value of 235 claimed by Buchanan & Anfinsen (1949) for their final preparation.

General properties of aconitase

The enzyme in dilute citrate buffer at pH 5.7 retained its activity for many weeks when stored in the frozen state and was unaffected by repeated freezing and thawing. It was not found necessary to add Fe²⁺ and
TABLE 2. Summary of Yields and Specific Activities of Fractions obtained during
the Purification of Acosintase from 2 kg. Pig Heart. (see text for details)

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Volume (ml.)</th>
<th>Protein Conc. (mg./ml.)</th>
<th>Total Protein (g.)</th>
<th>Units per ml.</th>
<th>Total Units</th>
<th>Specific Activity</th>
<th>Yield %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extract</td>
<td>6.240</td>
<td>6.3</td>
<td>39.3</td>
<td>70</td>
<td>437,000</td>
<td>11.1</td>
<td>100</td>
</tr>
<tr>
<td>1st. ethanol ppt.</td>
<td>832</td>
<td>6.35</td>
<td>5.3</td>
<td>310</td>
<td>260,000</td>
<td>49</td>
<td>60</td>
</tr>
<tr>
<td>2nd ethanol ppt.</td>
<td>112</td>
<td>19.6</td>
<td>2.2</td>
<td>1880</td>
<td>212,000</td>
<td>96</td>
<td>49</td>
</tr>
<tr>
<td>Dialyzed ammonium sulphate ppt.</td>
<td>116</td>
<td>8.4</td>
<td>0.98</td>
<td>1410</td>
<td>157,000</td>
<td>168</td>
<td>36</td>
</tr>
<tr>
<td>Supernatant after heating at 50°C</td>
<td>102</td>
<td>7.0</td>
<td>0.72</td>
<td>1320</td>
<td>135,000</td>
<td>188</td>
<td>31</td>
</tr>
<tr>
<td>pH 8.0 ethanol ppt.</td>
<td>25</td>
<td>16.5</td>
<td>0.41</td>
<td>3840</td>
<td>96,000</td>
<td>232</td>
<td>22</td>
</tr>
<tr>
<td>pH 8.5 ammonium sulphate ppt.</td>
<td>15</td>
<td>12.8</td>
<td>0.19</td>
<td>3360</td>
<td>50,500</td>
<td>265</td>
<td>12</td>
</tr>
</tbody>
</table>
cysteine as stabilizing agents, although in the absence of both or either of these agents the enzyme activity was markedly reduced. Details of the activation of aconitase will be reported in Chapter II. The final enzyme preparation could also be freeze-dried without loss of activity, but it was found more convenient to keep it in the frozen state. Fig. 2 shows that aconitase is stable to heat until a temperature of 51°C is attained, after which there is a sharp loss of activity. A characteristic feature of the enzyme was its wide range of precipitation. Aconitase activity was found in every fraction obtained with all solvents and salts used over the pH range from 5.7 to 8.5.

**Electrophoretic analysis**

The electrophoretic pattern of the final aconitase preparation is illustrated in Fig. 3. It was not possible to carry out electrophoretic analyses under conditions where the enzyme was on the acid side of its isoelectric point. All the components migrated to the anode when the electrophoresis was carried out at pH 8.6, but at pH 5.7 at least two of the minor components still migrated to the anode whilst the main component migrated to the cathode. pH values below 5.0 could not be used because of the denaturation of the enzyme.
Fig. 2. The effect of temperature on aconitase activity. Heating was carried out in a water-bath for 15 min., using 2.0 ml. samples of the dialysed ammonium sulphate precipitate (protein concentration, 9 mg./ml.). Aconitase activity was determined after activation with Fe$^{2+}$ and cysteine as described in the text. Temperature 30°; pH 7.4.
Fig. 3. Electrophoretic patterns of the aconitase preparation obtained after fractionation as described in the text. Veronal buffer, pH 8.6; ionic strength 0.1; temperature 1°C; current 10 milliamperes; protein concentration 1%; time 168 min.
Identification of the main electrophoretic component

In order to determine whether or not the main component was responsible for aconitase activity, a second electrophoretic run was carried out after dialysis of a 1% solution of the protein against veronal buffer at pH 8.6 (μ = 0.075) which contained sodium citrate (μ = 0.025). A similar electrophoretic pattern to that shown in Fig. 3 was obtained. Enzymic analysis of the main component after separation showed that this component was responsible for the aconitase activity, whilst the other components were devoid of activity. Both fractions were dialysed overnight against 0.004 M citrate buffer (pH 5.7) and activated with Fe^{2+} and cysteine before the aconitase activity was determined. Unfortunately, the enzyme lost activity during the dialysis and electrophoresis. The value obtained for the specific activity of the electrophoretically homogeneous protein was approximately half that of the original solution. The isolated main component was also capable of converting both cis-acetic acid and isocitric acid to citric acid.

The main component was completely free from the amber coloured pigment which was associated with the enzyme during the stages of purification. The mobility
of the amber coloured protein was somewhat similar to that of aconitase, so that electrophoresis had to be continued for a long period to effect separation. It may be calculated that the purity of aconitase in this preparation is 75-80%. If the main component consists entirely of aconitase, the fact that a 24-fold purification yields an enzyme preparation with a purity of 75-80% appears to indicate that aconitase represents a large proportion of the total protein of heart muscle. It is, of course, possible that the isolated component is not homogeneous in the ultracentrifuge, but this point has not yet been investigated.

Rates of citric acid formation from cis-aconitic and isocitric acids by aconitase fractions in the presence and absence of Fe$^{2+}$ and cysteine

Although the activation of aconitase is dealt with in Chapter II, the results of this aspect of the activation are given here as they are intimately concerned with the purification procedure.

During the purification, various fractions were tested for the ability to convert both isocitric acid and cis-aconitic acid to citric acid. Table 3 shows that the ratio of the two activities remains constant within
**TABLE 3.** Comparison of the Rates of Citric acid Formation from Isocitric acid and cis-Aconitic acid by Activated and Non-activated Fractions of Aconitase.

*Fractions were obtained and activated as described in the text*.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Non-activated</th>
<th>Activated</th>
<th>Ratio of Activities of enzyme</th>
<th>Specific Activities</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(a)</td>
<td>(b)</td>
<td>Ratio</td>
<td>(c)</td>
</tr>
<tr>
<td></td>
<td>cis-acon-</td>
<td>iso-cit-</td>
<td>a:b</td>
<td>cis-acon-</td>
</tr>
<tr>
<td></td>
<td>ic acid</td>
<td>ic acid</td>
<td></td>
<td>ic acid</td>
</tr>
<tr>
<td></td>
<td>(c)</td>
<td>(d)</td>
<td></td>
<td>(c)</td>
</tr>
<tr>
<td></td>
<td>(e)</td>
<td>(f)</td>
<td></td>
<td>(e)</td>
</tr>
<tr>
<td></td>
<td>(g)</td>
<td>(h)</td>
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<td>(g)</td>
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<tr>
<td></td>
<td>(i)</td>
<td>(j)</td>
<td></td>
<td>(i)</td>
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<tr>
<td></td>
<td>(k)</td>
<td>(l)</td>
<td></td>
<td>(k)</td>
</tr>
<tr>
<td></td>
<td>(m)</td>
<td>(n)</td>
<td></td>
<td>(m)</td>
</tr>
<tr>
<td>Extract</td>
<td>7.0</td>
<td>4.35</td>
<td>1.61</td>
<td>7.2</td>
</tr>
<tr>
<td>1st. ethanol ppt.</td>
<td>31</td>
<td>17.5</td>
<td>1.78</td>
<td>49</td>
</tr>
<tr>
<td>2nd. ethanol ppt.</td>
<td>50</td>
<td>32.5</td>
<td>1.54</td>
<td>96</td>
</tr>
<tr>
<td>Ammonium sulphate</td>
<td>72.5</td>
<td>45.5</td>
<td>1.59</td>
<td>151</td>
</tr>
<tr>
<td>ppt.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dialysed ammonium</td>
<td>7.0</td>
<td>4.7</td>
<td>1.49</td>
<td>142</td>
</tr>
<tr>
<td>sulphate ppt.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Supernatant after</td>
<td>6.7</td>
<td>4.2</td>
<td>1.60</td>
<td>174</td>
</tr>
<tr>
<td>heating at 50°</td>
<td></td>
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</tr>
</tbody>
</table>
the limits of experimental error. The addition of Fe²⁺ and cysteine increased the two activities equally so that there was no alteration in the ratio. The activating effect of Fe²⁺ and cysteine during the early stages of the purification was small and remained reasonably constant. After dialysis the activation was greatly enhanced. Fe²⁺ and cysteine can, therefore, replace completely the dialysable co-factors. The addition of 2,2'-dipyridyl (10⁻⁴ M) to the dialysis medium caused the formation of ferrous tridipyridyl inside the dialysis sac and this complex slowly diffused out into the medium. It is an interesting fact that not only does Fe²⁺ activate aconitase, but that loss of enzyme activity coincides with loss of Fe²⁺. It would thus appear likely that Fe²⁺ is the metal with which the enzyme is associated in vivo. The results also suggest that the Fe²⁺ is bound to the enzyme sufficiently strongly to resist splitting as a result of ethanol and ammonium sulphate fractionation, but the linkage is readily broken by dialysis.

Tests for the presence of other enzymes in the final aconitase preparation

The final aconitase preparation contained 156 units of isocitric dehydrogenase activity per mg. of protein
and 1100 units of fumarase' activity per mg. of protein. Tests for the presence of lactic dehydrogenase were negative. It is possible that two of the three minor components of the electrophoretic pattern are due to the presence of isocitric dehydrogenase and fumarase.

DISCUSSION

The inactivating effect of phosphate on aconitase in the absence of substrate can at least partially explain the instability of the enzyme as previously reported, for the tissues were extracted with phosphate buffer. It would seem that the apparent instability of the enzyme was related to the loss of co-factors, rather than to the denaturation of the apoenzyme. The final aconitase preparation was stable, but both it and the fractions obtained during the purification required the addition of Fe²⁺ and cysteine for maximum activity.

The assay figures of Johnson (1939) for the aconitase activity of various animal organs are also open to criticism as the organs were extracted with phosphate buffer. From the results obtained here, it would appear that the values obtained would depend on the time that elapsed between the extraction and the test of enzyme activity as well as the amount of substrate present in
the extract.

The possible mechanism for the inactivation of aconitase by phosphate in the absence of substrate will be discussed in Chapter III.

The fact that the relative rates of the reactions \( \text{cis-aconitic acid} \rightarrow \text{citric acid} \) and \( \text{isocitric acid} \rightarrow \text{citric acid} \) are the same throughout the fractionation procedure and that each reaction is activated to the same extent by \( \text{Fe}^{2+} \) and cysteine provides good evidence in favour of the idea that aconitase is a single enzyme. More conclusive is the finding that an electrophoretically homogeneous protein is capable of carrying out these reactions associated with aconitase activity. These findings are in accord with the fact that the loss of one enzyme activity invariably leads to the loss of all activities.

**SUMMARY**

1. The activation of aconitase by \( \text{Fe}^{2+} \) and cysteine has been confirmed. Phosphate was shown to inactivate the activated enzyme rapidly in the absence of substrate.

2. Aconitase has been purified 24-fold by low temperature
ethanol and ammonium sulphate fractionation. The purity of the final preparation was estimated to be 75-80%.

5. The final aconitase preparation was contaminated to a small extent by isocitric dehydrogenase and fumarase.

4. Aconitase has been isolated from the final preparation as an electrophoretically homogeneous protein. This protein was free of pigment and was capable of converting both isocitric acid and cis-aconitic acid to citric acid.

5. During the purification, the degree of activation of aconitase by Fe$^{2+}$ and cysteine increased, especially after dialysis.
CHAPTER II

THE ACTIVATION OF ACIDITAS
CHAPTER II

THE ACTIVATION OF ACONITASE

INTRODUCTION

Many enzymes exert their catalytic effects only in the presence of a metal or low molecular weight compounds. Some low molecular weight compounds, such as the phosphopyridine nucleotides and some metals, are considered to form complexes with the enzyme and are classified as coenzymes or prosthetic groups. Other low molecular weight compounds, such as cysteine and glutathione, activate certain enzymes by reaction, rather than by combination. One way in which this can happen is for these compounds to reduce disulphide linkages on the surface of those enzymes which require free -SH groups for activity. Thus, they differ from prosthetic groups and coenzymes in not taking part in the actual mechanism of catalysis and are better classified as activators. Glutathione is essential
for the activity of glyceraldehyde-3-phosphate dehydrogenase as shown by Arimsky & Racker (1952) and Racker & Arimsky (1952). But it is firmly bound to the enzyme and takes part in the reaction. Thus it must be classified in this instance as a prosthetic group rather than as an activator. As will be seen later, it would seem as though a number of prosthetic groups function by uniting the substrate with the enzyme through the active centre(s) and included in this group are some metal enzymes. Coenzymes appear to be linked to the enzyme at a site other than where the substrate is attached and act as acceptors of electrons or ions. Coenzymes are more highly dissociated than the prosthetic groups.

Green (1941) proposed the thesis that any substance which occurs in traces in the cell and which is necessary in traces in the diet of man or in the media for the growth of micro-organisms, must be an essential part of some enzyme system. This theory was based on the finding that a number of trace substances had been found to exert their effects either as prosthetic groups or coenzymes of enzymes. It, therefore, gave a rational basis for the explanation of how trace substances exert such profound biological effects. The small amounts of these substances required were
consistent with the fact that the concentration of particular enzymes in the cell is low. This hypothesis has been strengthened as a result of further research over the intervening years. It has also come to light that many enzymes have a specific requirement for a particular coenzyme or prosthetic group, and this has led to a classification of enzymes according to the structure of these compounds. The fact that different enzymes require the same co-factor, although they have mutually exclusive specificities, indicates that the protein moiety is not only specific, but also determines the nature of the catalysis.

Although it is likely that the explanation of the requirement of an organism for trace metals is or will be found to be due to their role in enzyme systems, it is not only the trace metals that serve as prosthetic groups or activators. Of the so-called bulk metals (K, Na, Ca, Mg and Fe), all with the possible exception of Na are capable of activating enzymes. Other metals such as V and Nb have also been found in animal and plant tissues, but at the present time it is not known whether or not they participate in enzymic reactions.

The exact function of the metal in such enzymes
systems is not clear. In certain cases, the catalytic activity of the metalloenzyme is manifested to some extent in the metal itself, but the addition of the apoenzyme together with the metal greatly enhances the reaction rate. In such cases, it is considered that the function of the apoenzyme is to enhance the inherent properties of the metal. An example of this type of reaction is found with the oxidative decarboxylation of \( \alpha \)-ketoglutaric acid. \( \alpha \)-Ketoglutaric carboxylase requires \( \text{Zn}^{2+} \) for activity, but Kalinitsky (1953) has shown that the same reaction occurs in the presence of \( \text{Zn}^{2+} \) and in the absence of the apoenzyme, although the reaction rate is slower in the absence of the apoenzyme. This cannot be the function of the apoenzyme in the case of all metalloenzymes. Although \( \text{Zn}^{2+} \) is essential for the activity of carbonic anhydrase, Eilkin & Mann (1940) showed that inorganic zinc salts, simple and complex organic zinc compounds were devoid of catalytic activity. The function of the \( \text{Zn}^{2+} \) here might be to unite the enzyme and substrate in a coordination complex as suggested by Smith (1951), rather than to increase the ability of \( \text{Zn}^{2+} \) to catalyse the reaction. It is, of course, possible that \( \text{Zn}^{2+} \) does have some catalytic
activity, but it is so low that it cannot be measured.

Smith (1951) found that the addition of a peptidase capable of hydrolysing L-leucylglycine to Mn^{2+} caused striking changes in the absorption spectrum of the metal. When L-leucylglycine was added to Mn^{2+}, absorption changes were also observed. These findings led Smith to postulate the formation of an enzyme-substrate complex which involved co-ordination complexes of Mn^{2+}. He proposed that after the formation of the enzyme-substrate complex, the decrease in the activation energy of hydrolysis was produced by attraction of the electrons by Mn^{2+} while the protein centre oriented the substrate so that the net effect was an electronic deformation of the sensitive bond. The hydrolysis was then considered to be due to the catalytic effect of H^+ and OH^- ions.

In the absence of further information, it would seem reasonable to assume that a metal unites with the apoenzyme to form an active complex which is then in equilibrium with the free apoenzyme and free metal according to the equation:

\[
\text{metal + enzyme} \rightleftharpoons \text{metal-enzyme} \\
\text{(inactive)} \quad \text{(active)}
\]
From such an equation, it is possible to determine the hypothetical dissociation constant of the complex. This has been done for many enzymes with a result that it has been found that the affinity of apoenzymes for metal varies from system to system and within the same system for different metals. Dialysis studies have also shown this difference in affinity. Smith & Hanson (1949) found that the Mg$^{2+}$ of carboxypeptidase is not removed by dialysis, whereas the Mg$^{2+}$ of leucine aminopeptidase is readily removed by dialysis. Although enzymes vary as to their affinity for metal ions, after dialysis the metal concentration required for the enzyme to show its maximum activity is relatively low.

Not all enzyme systems show an absolute metal specificity. A number of phosphatases, phosphate transferring enzymes, and $\alpha$-keto-acid carboxylases can be activated by either Mn$^{2+}$ or Mg$^{2+}$, although Mn$^{2+}$ is more effective than Mg$^{2+}$, whilst other metals of the transition series are inactive. This indicates that the ability of a metal ion to activate enzyme systems is not simply related to the properties of the metal ion. The difference between Mn$^{2+}$ and Mg$^{2+}$ with respect to mass, ionic radius, ionic potential, mobility
and rate of diffusion is considerably greater than between Mn$^{2+}$ and the other transition metals. It is probable that hydration and complex formation are more concerned with the ability of a metal to activate an enzyme system, than are the other physical properties. At the same time, it is possible that the properties of the 'naked' metallic ion may differ greatly from those which it possesses in a physiological medium where it is surrounded by other molecules of an inorganic and organic nature (Lehniger, 1959).

The activation of some metal enzymes has been found to be a very slow reaction. Mohamed & Greenberg (1945) found that the activation of arginase was dependent on time as well as other factors. Under certain conditions, days were required for the enzyme to be fully activated. Smith (1951) also found a similar slow rate of activation with peptidases. This slow rate of activation may be significant and find explanation in the time required for the formation of a co-ordination compound between the metal and the enzyme, but it is noteworthy that none of the enzymes known to show a very slow rate of activation has been obtained as an electrophoretically homogeneous protein.
Dicusman & Cloutier (1951) showed that aconitase was a metalloenzyme which was dependent on the presence of Fe$^{2+}$ and a reducing agent for maximum activity. Qualitative studies showed that Fe$^{2+}$ was capable of forming complexes with the substrates of aconitase under the conditions of optimum enzyme activity and the results also suggested that the Fe$^{2+}$ was capable of forming a complex with the aconitase. These authors suggested that the Fe$^{2+}$ was responsible for the linkage between the aconitase and the substrate in the aconitase-tricarboxylic acid complex. It was assumed that the reducing agents played a double role in maintaining the iron as Fe$^{2+}$ and in keeping the reducing groups of the protein in the reduced state, but no evidence was presented to indicate that this was the case.

It has been seen that some metal ions are capable of activating some enzymes; different theories to explain the function of the metal have been put forward, but the mode of activation has not yet been definitely established. Dicusman & Cloutier (1951) explained their results concerning aconitase in terms of the hypothesis advanced by Smith (1951). Whilst their results were not inconsistent with this hypothesis, the authors
provided little or no direct evidence to show that the function of Fe\textsuperscript{2+} was to form a link between the enzyme and the substrate. No evidence was presented to indicate the role played by the reducing agents in the activation of the enzyme. The high residual activity of the Dickman & Cloutier aconitase preparation in the absence of Fe\textsuperscript{2+} and reducing agents did not permit of a more detailed investigation of the function of these compounds in the aconitase system. The aconitase preparation obtained by the method reported in Chapter I provided a much better means of determining whether these compounds functioned as prosthetic groups, activators or both. The preparation showed little activity in the absence of Fe\textsuperscript{2+} and a reducing agent, whereas it showed considerable activity on the addition of these substances. This preparation was used for testing the effect of Fe\textsuperscript{2+} and reducing agents on aconitase activity. The results reported in this chapter indicate that both Fe\textsuperscript{2+} and reducing agents act as prosthetic groups of aconitase and so are concerned in the formation of the active enzyme complex. It is suggested that the active form of the enzyme is an enzyme-Fe\textsuperscript{2+}-reducing agent complex.
EXPERIMENTAL

Methods

Aconitase was activated by Fe$^{2+}$ and all reducing agents in the same fashion as previously described (page 19). The enzyme solutions contained 60 μg of the final aconitase preparation/ml. The enzyme activity was determined and citric acid estimated as previously described (page 20).

Reagents

Ascorbic acid was a Roche product, thioglycollate was a B.D.H. product which was re-distilled before use and glutathione was a gift from the Distillers Co. Ltd., Liverpool. As determined by thiol titration, the glutathione was 99½ pure. All these compounds were free from iron. The other reagents were either A.R., or as previously mentioned.

RESULTS

Activation of aconitase by Fe$^{2+}$ and cysteine

Fig. 4 shows the effect of Fe$^{2+}$ and cysteine on the activity of aconitase. The final purified, dialyzed preparation possesses little activity; cysteine alone
Fig. 4. Activation of aconitase by Fe$^{2+}$ and cysteine.

Aconitase was incubated for 1 hr. at 0$^\circ$ and pH 7.4 before the activity was determined as described in the text.

- No additions
- 5 x 10$^{-4}$ M Fe$^{2+}$
- 0.01 M cysteine
- 5 x 10$^{-4}$ M Fe$^{2+}$ + 0.01 M cysteine
does not increase the activity; Fe$^{2+}$ alone brings about a 15-fold increase in the activity, whilst the addition of Fe$^{2+}$ and cysteine increases the activity 70-fold. The experiments reported in Chapter I had shown that cysteine alone would activate aconitase preparations, but later this was shown to be due to the presence of Fe$^{2+}$ in the cysteine preparation. The investigation of the activation of aconitase by Fe$^{2+}$ alone involved some technical difficulties. On account of the small amount of protein present in solution, it showed little or no buffering capacity. The ferric ammonium sulphate solution was acidic so that if it were not partly neutralised the pH of the Fe$^{2+}$-enzyme solution was below pH 5.0 with a result that there was denaturation of the enzyme. The adjustment of the pH of this solution to 7.4 was difficult and at this pH value at least some of the Fe$^{2+}$ was in the colloidal state. Therefore, it is not possible to state that the above value represents an accurate estimation of the activation of aconitase by Fe$^{2+}$. Nevertheless, it is clear that Fe$^{2+}$ and cysteine bring about a very marked activation of aconitase.

Time required for maximum activation of aconitase by
Fe$^{2+}$ and cysteine

The conditions chosen in the previous experiments for the determination of aconitase activity were based on those of Dickman & Cloutier (1951). Although they were quite arbitrary, they provided a suitable means for following the purification of the enzyme. But having obtained a highly purified preparation, it was important to determine whether or not these conditions gave rise to maximum activation. The results of Dickman & Cloutier (1951) suggested that an interaction between the apoenzyme and the Fe$^{2+}$ to form a Fe$^{2+}$-enzyme complex according to the equation:

$$\text{Fe}^{2+} + \text{enzy} \rightarrow \text{Fe}^{2+}-\text{enzy}$$

was essential for enzyme activity. It was possible that the rate of the attainment of the equilibrium was slow and that the 1 hr. period of activation of the enzyme with Fe$^{2+}$ and cysteine was not sufficiently long for the enzyme to be activated to the full extent. Therefore, an investigation was made of the relationship between the enzyme activity and the period of incubation.

It is clear from Table 4 that there is an initial rapid activation of aconitase by Fe$^{2+}$ and cysteine, followed by a slower period of activation, so that about
### TABLE 4

**Effect of Pre-incubation Time on the Activation of Aconitase by Fe$^{2+}$ and Cysteine**

(Aconitase was incubated at pH 7.4 and 0°C with $5 \times 10^{-4} \text{ M}$ Fe$^{2+}$ and $10^{-2} \text{ M}$ cysteine. 0.2 ml. samples, equivalent to 12 µg. of the final aconitase preparation, were removed at intervals and added to test tubes containing 0.5 ml. of cis-aconitic acid (final concentration $4 \times 10^{-3} \text{ M}$) at pH 7.4 and 4.3 ml. of water. Reaction was stopped after 15 min. by the addition of 0.5 ml. of 50% (w/v) trichloracetic acid and the mixture analysed for citric acid. Temperature 30°C.)

<table>
<thead>
<tr>
<th>Time of activation (hr.)</th>
<th>Aconitase activity ($\mu$g. citric acid formed/15 min.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>265</td>
</tr>
<tr>
<td>0.5</td>
<td>335</td>
</tr>
<tr>
<td>1.0</td>
<td>320</td>
</tr>
<tr>
<td>2.0</td>
<td>320</td>
</tr>
<tr>
<td>3.0</td>
<td>320</td>
</tr>
<tr>
<td>4.0</td>
<td>318</td>
</tr>
<tr>
<td>5.0</td>
<td>295</td>
</tr>
<tr>
<td>6.0</td>
<td>278</td>
</tr>
<tr>
<td>24.0</td>
<td>192</td>
</tr>
</tbody>
</table>
30 min. at $0^\circ$ is required for maximum activation. This activity is retained for at least 4 hr., after which there is a slow decrease so that at 24 hr., the enzyme possesses only 60% of its maximum activity. These results indicate that the arbitrary conditions previously chosen for the incubation do bring about maximum activation of the enzyme.

There seemed to be three possible reasons by which the loss of enzyme activity after 4 hr. could be explained. They were:

(a) alteration of the pH of the mixture during the incubation period at $0^\circ$

(b) accumulation of the end-products of the oxidation of cysteine by Fe$^{2+}$

(c) denaturation of the enzyme in dilute solution.

A check of the pH of the mixture during the incubation showed that there was no alteration over the 24 hr. period. The accumulation of the end-products of cysteine oxidation could not explain the loss of activity, for when the enzyme was added to a mixture of Fe$^{2+}$, cysteine and water which had been incubated at $0^\circ$ for 24 hr., the activity following a 30 min. incubation period at $0^\circ$ was maximal. It is unlikely that there is any significant accumulation
of the end-products of the oxidation of cysteine, for although no special precautions were taken to exclude oxygen, the conditions were virtually anaerobic. It must be concluded that the loss of enzyme activity is due to the denaturation of the enzyme in dilute solution.

Activation of aconitase by the addition of Fe$^{2+}$ and cysteine to the test medium

With the previous method of activating aconitase with Fe$^{2+}$ and cysteine, it was assumed that there was no dissociation of the active complex when it was added to the test medium containing substrate. Whilst the concentrations of Fe$^{2+}$ and cysteine in the enzyme solution during the period of activation were $5 \times 10^{-4}$ M and $10^{-2}$ M respectively, when the enzyme solution was added to the test medium the concentrations were reduced to $10^{-5}$ M and $2 \times 10^{-4}$ M. Dissociation of the enzyme complex was likely on account of the high dilution, so an investigation was made of the influence of the Fe$^{2+}$ and cysteine concentration in the test medium on the aconitase activity. In these experiments, the activation was carried out at 30° for convenience.

It was found that when the non-activated aconitase was added to a medium containing the lower concentrations
of Fe$^{2+}$ and cysteine and incubated for 5 min. at 30°
before the addition of substrate, the enzyme showed no
activity. When the concentrations of Fe$^{2+}$ and cysteine
in the medium were increased to $5 \times 10^{-4}$ M and $10^{-2}$ M
respectively, the enzyme showed the same activity over
the initial period of the reaction as it did when the
original method of activation was used. (The activa-
tion of aconitase in the presence of $5 \times 10^{-4}$ M Fe$^{2+}$
and $10^{-2}$ M cysteine in the test medium will be designated
as Method II. The original method of activation, i.e.,
the activation of the enzyme in the presence of $5 \times 10^{-4}$ M
Fe$^{2+}$ and $10^{-2}$ M cysteine so that a sample of this
solution is added to the test medium, will be referred
to as Method I). Fig. 5 shows the effect of adding
the enzyme directly to a mixture of Fe$^{2+}$, cysteine and
substrate. It will be noted that there is a distinct
lag period of about 3 min. and that the reaction rate
is slower than that of the control in which the enzyme
was pre-incubated with Fe$^{2+}$ and cysteine for 5 min.
prior to the addition of substrate.

It may be concluded from these results that
dissociation of the enzyme complex does not occur on
dilution, at least in the presence of substrate. The
Fig. 5. The activation of aconitase by the addition of Fe$^{2+}$ and cysteine to the test medium. 12 μg. of the non-activated aconitase preparation were added to the test medium which contained $5 \times 10^{-4}$ M Fe$^{2+}$, $10^{-2}$ M cysteine and $4 \times 10^{-3}$ M cis-aconitic acid. Total volume 5.0 ml., temperature 30°, no buffer, pH 7.4.

- aconitase incubated 5 min. with Fe$^{2+}$ and cysteine before the addition of substrate
- aconitase added to a mixture of Fe$^{2+}$, cysteine and substrate without pre-incubation.
fact that a finite time is required for maximum activation of the enzyme is verified, but at 30° the period is greatly reduced. If it is assumed that the rate of activation of aconitase is doubled for each 10° rise in temperature, an activation period of 30 min. at 0° is equivalent to 3.7 min. at 30°, so the results are in reasonable agreement.

**Activation of aconitase under aerobic and anaerobic conditions**

The fact that a reducing agent was required to obtain maximum activation of aconitase suggested that higher enzyme activities might be obtained under strictly anaerobic conditions. Experiments showed that there was no increased enzyme activity when the activation and test were carried out under nitrogen. This was not altogether surprising in view of the fact that the usual test conditions were essentially anaerobic. Oxygen was admitted to the solution only during the time of mixing and apparently the relatively large amount of cysteine present was adequate to prevent oxidation of the Fe²⁺ or enzyme taking place.

If the Fe²⁺-cysteine-aconitase mixture was rapidly shaken in air during the activation period, the enzyme
showed no activity on the addition of the substrate. Possibly this was due to the complete oxidation of cysteine in the presence of Fe\(^{2+}\), with a result that the cysteine concentration fell to a level at which it was unable to participate in the activation of aconitase. It was also possible that the insoluble cysteine produced as a result of the oxidation of cysteine could have adsorbed the enzyme thus preventing the activation taking place, or alternatively rendering the enzyme inactive as a result of the adsorption taking place through the active centre(s) of the enzyme.

**Effect of shaking activated aconitase rapidly in air**

It was of interest to determine whether or not activated aconitase could be inactivated by oxygen during the course of the reaction. The aconitase was activated by both methods I and II, substrate was added and the reaction rates determined with and without shaking. Fig. 6 shows that there is a slight loss of enzyme activity when the test medium is rapidly shaken in air, following activation by method I. On the other hand, there is a marked loss of activity under these conditions when the activation is carried out by method II. A comparison of the two controls, which were not
Fig. 6. The effect of rapid shaking in air on the activity of activated aconitase. The amount of aconitase added was equivalent to 12 µg. of the final aconitase preparation. Temperature 30°, no buffer pH 7.4.

- aconitase activated by method I as previously described (page 19)
- aconitase activated by method II as described in Fig. 5.

--- non-shaken  ...... shaken
shaken, shows that the initial rates are identical, but in the case of aconitase activated by method II, there is a small loss of activity after 10 min. This was a characteristic feature of this means of activation.

Three possible reasons to account for the inactivation of aconitase under these conditions are:

(a) Oxidation of essential -SH groups on the enzyme surface

(b) Formation of $Fe^{3+}$ which is inhibitory

(c) Formation of cystine which is inhibitory.

As the inactivation of the enzyme is greater in the presence of larger amounts of cysteine, (a) and (b) cannot explain the inhibition. The cysteine concentration at any one time must be greater when the enzyme is activated by method II than it is when the enzyme is activated by method I, so that the degree of reduction of the iron and enzyme must be greater. The addition of cystine to the medium in an amount less than that required for a saturated solution did not cause any inhibition. Although cystine in solution did not inhibit aconitase, the adsorption of aconitase by suspended cystine could explain the inhibition. This idea is consistent with the finding that the presence of
high concentrations of cysteine which give rise to suspended cystine as a result of oxidation, caused more marked inhibition of aconitase. The loss of activity in the control of the enzyme activated by method II can also be explained by the formation of smaller amounts of cystine when the solution is not shaken.

These results are of importance as far as artificial systems for the oxidation of citric acid are concerned. Activation of aconitase by method I, so that the concentrations of Fe$^{2+}$ and cysteine in the test medium are minimal, is clearly preferable if the aconitase is to act at a maximum rate for long periods of time under aerobic conditions.

**Activation of aconitase by reducing agents**

Dickman & Cloutier (1951) had shown that ascorbic acid was as effective as cysteine in activating aconitase, whereas glutathione was only about half as effective. As the preliminary results obtained with the more highly purified preparation of aconitase differed from those of these authors, the ability of reducing agents to activate aconitase was re-investigated.

Table 5 shows the relative effectiveness of four reducing agents in activating aconitase in the presence
TABLE 5

**Effect of Reducing Agents on Aconitase Activity**

Aconitase was incubated with the reducing agent and 5 x 10^{-4} M Fe^{2+} for 1 hr. at 0^\circ. 0.25 ml. samples, equivalent to 15 μg. of the final aconitase preparation, were added to media containing 1.5 ml. of 0.1 M phosphate buffer (pH 7.7), 0.5 ml. of cis-aconitic acid (final concentration 6.7 x 10^{-3} M) and 0.75 ml. of water. Reaction was stopped after 15 min. by the addition of 0.5 ml. of 50% (w/v) trichloroacetic acid and the mixture analysed for citric acid. Temperature 22^\circ.

<table>
<thead>
<tr>
<th>Reducing agent</th>
<th>Aconitase Activity (μg. citric acid formed)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.01 M</td>
</tr>
<tr>
<td>Cysteine</td>
<td>178</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>100</td>
</tr>
<tr>
<td>Glutathione</td>
<td>23</td>
</tr>
<tr>
<td>Thio-glycollate</td>
<td>129</td>
</tr>
</tbody>
</table>
of a fixed amount of \( \text{Fe}^{2+} \). At a level of 0.01 M, cysteine brings about the greatest activation of aconitase, whilst thioglycollate, ascorbic acid and glutathione are less effective in that order. It was surprising that glutathione activated the highly purified enzyme to such a small extent, but later experiments showed that the activation was increased when higher concentrations of glutathione were used. In view of this result, the experiment was repeated with the reducing agents at concentrations of 0.01 M and 0.025 M and both results are included here for comparative purposes. It is seen that when the level of the reducing agents is increased to 0.025 M, the degree of activation is increased in all cases. Cysteine is still the most effective, but the activation by glutathione is increased to such an extent that it is as effective as thioglycollate. The higher concentration of ascorbic acid caused only a small increase in the enzyme activity, so that at the higher level, it was least effective.

**Specificity of \( \text{Fe}^{2+} \) in the activation of aconitase**

As \( \text{Fe}^{2+} \) was markedly catalytic in the oxidation of cysteine, as first shown by Mathews & Walker (1909), it was of interest to determine whether or not these metals
which are capable of oxidising cysteine are also capable of activating aconitase. It was known that Mn²⁺ and Cu²⁺ are also capable of catalytically oxidising cysteine, so these metals were tested for ability to activate aconitase. No other metals are known to oxidise cysteine catalytically.

It was found that Cu²⁺ and Mn²⁺ at a concentration of 5 x 10⁻⁴ M did not bring about any activation of aconitase. Moreover, when this concentration of Cu²⁺ was added to the usual activation system containing Fe²⁺ and cysteine, the activity of the enzyme was inhibited 50%. These results confirm and extend those of Krebs & Eggleston (1944). On the other hand, Mn²⁺ was without effect. Thus it would seem as though there was no relationship between the ability of a metal to oxidise cysteine catalytically and its ability to activate aconitase. Results also indicated that there was a lack of any correlation between the other reducing agents and the metals capable of oxidising them catalytically, and the ability of these systems to activate aconitase. It is worthy of note that Cu²⁺ and Mn²⁺ which belong to the same transition series as Fe²⁺ and which therefore have similar properties, do not activate aconitase.
This indicates the high specificity of aconitase for Fe$^{2+}$. No other metals were tested as activators of aconitase, but it is clear from the work of Dickman & Cicutier (1950) that Fe$^{2+}$ is specific in this regard.

**Role of Fe$^{2+}$ in the action of aconitase**

In order to elucidate the role played by Fe$^{2+}$ in the action of aconitase, a quantitative study was made of the effect of the Fe$^{2+}$ concentration on the activity of aconitase.

The observations on the relationship between the Fe$^{2+}$ concentration and the reaction velocity in the presence of cysteine and ascorbic acid, as shown in Fig. 7, conform to a simple Michaelis-Menten relation i.e. to the equation $v = \frac{Vx}{K_x + x}$ where $v$ = initial velocity of the reaction at a standard concentration of substrate, in the presence of a reducing agent; $x$ = concentration of Fe$^{2+}$, and $K_x$ = hypothetical dissociation constant of the Fe$^{2+}$-enzyme complex formed according to the equation

$$Fe^{2+} \text{enzyme} \rightleftharpoons Fe^{2+} \text{-enzyme}$$

These results are consistent with the hypothesis that Fe$^{2+}$ combines with aconitase in the ratio of one Fe$^{2+}$ ion to one active centre to form the Fe$^{2+}$-enzyme complex which
Fig. 7. The effect of Fe$^{2+}$ concentration onaconitase activity. Aconitase was activated in the presence of $10^{-2}$ M cysteine or ascorbic acid and various concentrations of Fe$^{2+}$ at pH 7.4 for 1 hr. at 0°. 0.25 ml. samples of the enzyme solutions, containing 15 µg. of the final aconitase preparation, were added to media containing 1.5 ml. of 0.1 M phosphate buffer (pH 7.7), 0.5 ml. of neutralised cis-aconitic acid (final concentration $6.7 \times 10^{-3}$M) and 0.75 ml. of water. The reaction was stopped after 15 min. by the addition of 0.5 ml. of 50% (w/v) trichloroacetic acid and the solutions analysed for citric acid. Temperature 22°.  

- $S$ = molar conc. of Fe$^{2+} \times 10^4$  
- $c$ ascorbic acid  
- $V$ = µg. of citric acid formed/15 min.$ \times 10^{-3}$  
- cysteine
is responsible for enzymic activity.

This interpretation is valid only because it was known from previous experiments that the above equilibrium was established during the pre-incubation of the enzyme with Fe$^{2+}$ and the reducing agent for 1 hr. at 0°. As the substrate was not added and the reaction rate was not determined until after the equilibrium was established, it can be taken that the reaction rate was proportional to the amount of the Fe$^{2+}$-enzyme complex formed. As the final enzyme preparation was subjected to prolonged dialysis against citrate buffer (pH 5.7) before use, it was assumed that there was little or no Fe$^{2+}$ remaining in the preparation. It was on this basis that the hypothetical dissociation constant was calculated to be $3.9 \times 10^{-6}$ M in the presence of cysteine and $1.7 \times 10^{-5}$ M in the presence of ascorbic acid. The above assumption may not be correct in view of the fact that the enzyme did possess slight residual activity in the absence of Fe$^{2+}$ and a reducing agent. On this account the value obtained may not be correct and may also vary from one preparation to another. However, it is clear thataconitase has a very high affinity for Fe$^{2+}$.

It can also be seen from fig. 7 that the absolute
value of the maximum rate obtained in the presence of
cysteine is over twice as great as that obtained in the
presence of ascorbic acid. If the active complex is
of the type $Fe^{2+}$-enzyme, then when the $Fe^{2+}$ conцentra-
tion rises sufficiently, the limiting concentration of
the $Fe^{2+}$-enzyme complex obtained must be independent of
the nature of the reducing agent. But the maximum
velocity is dependent on the nature of the reducing
agent which indicates that the reducing agent is concerned
in the formation of the active complex. This point
will be discussed further in a later section.

Reaction of $Fe^{2+}$ with reducing agents

From the colour of the enzyme solutions during
the activation period, it was clear that $Fe^{2+}$ had
reacted with cysteine, thioglycollate, ascorbic acid
and glutathione to form coloured complexes. Schubert
(1932) and Barron (1951) had shown that cysteine reacts
with $Fe^{2+}$ at alkaline pH values to form a complex of
type (I)

\[ \text{I} \]

\[ \text{II} \]
which can combine with CO to form a stable colourless complex, or with oxygen to form an unstable blue-violet complex of type (II). A blue-violet colour formed when the enzyme solution containing \( \text{Fe}^{2+} \) and cysteine was shaken, but faded on standing. A stable cherry-red complex formed as a result of the reaction between \( \text{Fe}^{2+} \) and thioglycollate. Schubert (1942) showed that this complex was also of type (I). A violet complex formed between \( \text{Fe}^{2+} \) and ascorbic acid, but no evidence for the structure of this complex could be found in the literature. However, the structure of ascorbic acid would seem to permit of the formation of a complex with \( \text{Fe}^{2+} \). Glutathione and \( \text{Fe}^{2+} \) gave a stable golden yellow complex, but again no evidence could be found for the structure of this complex. Whereas the \( \text{Fe}^{2+} \)-cysteine complex was coloured only in the presence of oxygen, the other complexes were coloured in the absence of oxygen.

**Equilibrium reactions of the aconitase system**

The experiments to date had indicated that reducing agents as well as \( \text{Fe}^{2+} \) were concerned in the formation of the active aconitase complex. The equilibrium between the enzyme, \( \text{Fe}^{2+} \) and cysteine might be as follows:
\[
\text{enzyme + cysteine} \xrightarrow{\text{Fe}^{2+}} \text{enzyme-cysteine} \uparrow \text{Fe}^{2+} \downarrow \text{Fe}^{2+} \\
\text{Fe}^{2+} (\text{cysteine})_2 \xrightarrow{\text{Fe}^{2+}} (\text{enzyme-cysteine})
\]

so that the \( \text{Fe}^{2+} (\text{enzyme-cysteine}) \) complex was the active complex in the case of cysteine. If the affinity of the enzyme for cysteine were greater than the affinity of \( \text{Fe}^{2+} \) for cysteine, it might be expected that high concentrations of cysteine would give rise to maximum formation of the enzyme-cysteine complex. At the same time, the enzymic activity could be reduced as a result of the equilibrium between the \( \text{Fe}^{2+} \) and cysteine being displaced in favour of the formation of the \( \text{Fe}^{2+} (\text{cysteine})_2 \) complex. The same argument might also be expected to apply in the case of ascorbic acid. Fig. 8 shows that this is the case with both reducing agents. It is clear also that the enzyme activity in the presence of cysteine is greater than in the presence of ascorbic acid. The similarity of the curves is more apparent when the enzyme activities are plotted as percentages of the activity in the presence of the reducing agent at a concentration of \( 10^{-2} \) M as shown in Fig. 9. These results are consistent with the idea that the activation processes are similar
Fig. 8. The effect of increasing concentrations of cysteine and ascorbic acid on aconitase activity. Aconitase was incubated with various concentrations of cysteine or ascorbic acid and $5 \times 10^{-4} M \text{Fe}^{2+}$ for 1 hr. at $0^\circ$ and pH 7.4. Activity determinations were carried out as described in fig. 7.

○ = cysteine \hspace{1cm} ● = ascorbic acid
Fig. 9. The effect of increasing concentrations of cysteine and ascorbic acid on asmitase activity. Conditions as described in fig. 8.

○ = cysteine    ● = ascorbic acid
with both cysteine and ascorbic acid, the absolute activity of the active complex being dependent on the nature of the reducing agent.

Role of reducing agents in the action of aconitase

In the hope of establishing more securely the hypothesis that the active form of aconitase is a complex containing cysteine, ascorbic acid or other activators, an investigation was made of the effect of varying concentrations of the reducing agents on the aconitase activity in the presence of $5 \times 10^{-4}$ M Fe$^{2+}$.

Cysteine, thioglycollate and ascorbic acid

The observations on the relationship between the concentration of cysteine, thioglycollate and ascorbic acid and the reaction velocity, as shown in fig. 10, conform to a simple Michaelis-Menten relation, i.e., to the equation

$$v = \frac{V_0}{K_c + c}$$

where

$v =$ initial velocity of the reaction at a standard concentration of substrate, in the presence of $5 \times 10^{-4}$ M Fe$^{2+}$.
$c =$ concentration of the reducing agent
$K_c =$ hypothetical dissociation constant of the reducing
Fig. 10. The effect of cysteine, thioglycollate and ascorbic acid concentration on ascoritase activity in the presence of Fe$^{2+}$. Ascoritase (50 μg. of the final preparation/ml.) was incubated with $5 \times 10^{-4}$ M Fe$^{2+}$ and various concentrations of the reducing agents for 1 hr. at 0° and pH 7.4. The enzyme activity was determined as described in Fig. 7.

$s = \text{molar concentration of reducing agent } \times 10^2$

$V = \text{μg. of citric acid formed/15 min. } \times 10^{-3}$

$x$ cysteine   ○ ascorbic acid   ● thioglycollate
agent-enzyme complex, formed according to the equation

enzyme + reducing agent ⇌ enzyme-reducing agent

There was a tendency for the points obtained at low concentrations of the reducing agents to lie off a straight line; this fact may have some significance, but the experimental error was much greater with the low reaction rates. The results indicate that each of the reducing agents combines with aconitase in the ratio of one molecule of the reducing agent to one active centre to form an enzyme-reducing agent complex.

However, this complex cannot be considered in itself as an active complex in view of the previous finding that the enzyme is inactive in the presence of a reducing agent alone. No activities were obtained unless Fe^{2+} was also present. Again this interpretation is valid only because the system had attained equilibrium before the reaction rates were determined.

The hypothetical dissociation constants of the enzyme-reducing agent complex were calculated to be $3.6 \times 10^{-5}$ M for cysteine, $1.2 \times 10^{-3}$ M for ascorbic acid and $6.0 \times 10^{-5}$ M for thioglycollate. These values indicate that the affinity of the enzyme for ascorbic
acid > cysteine > thioglycollate. There is no relationship between the affinities of the enzyme for the reducing agents and the maximum activity of the enzyme in the presence of the same reducing agents. Cysteine gives the greatest maximum velocity, ascorbic acid gives the lowest, whilst thioglycollate gives an intermediate maximum velocity value. These findings are in keeping with the idea that the active form of the enzyme is the complex reducing agent-enzyme-Fe$^{2+}$, the properties of the complex being determined by the nature of the reducing agent.

The plot of the reciprocal of the velocity against the reciprocal of the square of the reducing agent concentration gave a curve rather than a straight line. If the reducing agents formed a Fe$^{2+}$(reducing agent)$_2$ complex which combined with the enzyme, or if the active form of the enzyme was enzyme-(reducing agent)$_2$, then this plot would give a straight line. Although Fe$^{2+}$ forms complexes of the above type with the reducing agents, it is not these complexes which are responsible for the activation of aconitase.

Glutathione

When glutathione was tested for its ability to
activate aconitase. It was seen that very little activation was obtained at a concentration of 0.01 M. However, higher concentrations of glutathione gave an appreciable activation of the enzyme; maximum activation was obtained with glutathione at a concentration of 0.025 M. Having found that aconitase could be activated by glutathione, a study was made of the effect of glutathione concentration on the enzyme activity. Fig. 11 shows that there is no simple relationship between the concentration of glutathione and the enzyme activity, such as was found with cysteine, thiosulfate and ascorbic acid. The fact that a curve is obtained when the reciprocal of the velocity is plotted against the reciprocal of the glutathione concentration would seem to indicate that the mechanism of the activation is different from that obtained with the other reducing agents.

DISCUSSION

The marked activation of aconitase by Fe$^{2+}$ in the presence of a reducing agent and the failure of other metal ions to activate, is indicative of Fe$^{2+}$ being a specific integral component of the aconitase system.
Fig. 11. The effect of glutathione concentration onaconitase activity. The conditions were the same as those described in Fig. 10.

\[ S = \text{molar concentration of glutathione} \times 10 \]
\[ V = \mu g. \text{ of citric acid formed} / 15 \text{ min.} \times 10^{-3} \]
The observations on the relationship between the Fe$^{2+}$ concentration and the enzyme activity are consistent with the idea that Fe$^{2+}$ reacts with the enzyme in the ratio of one Fe$^{2+}$ ion to one active centre of the enzyme according to the equation

$$Fe^{2+} + \text{enzyme} \rightleftharpoons Fe^{2+}\text{-enzyme}$$

Thus the Fe$^{2+}$-enzyme complex can be considered as an active component of the system. As the activation of the enzyme is increased by the presence of a reducing agent and influenced by the type of reducing agent, this cannot be the only reaction concerned in the activation.

The equilibrium between the enzyme and the activators is not established immediately; a definite time is required before the enzyme attains its maximum activity. This suggests that the free energy of the activation is significant. Thus the interaction cannot be entirely electrostatic as such a reaction is instantaneous and does not require activation energy.

The four reducing agents tested have the ability of increasing the enzyme above that obtained with Fe$^{2+}$ alone, although they are not equally effective. The function of the reducing agents could be related to their
ability to maintain the iron as $Fe^{2+}$. Since the concentrations of the reducing agents were always in excess of that of $Fe^{2+}$ and the oxidation-reduction potentials are such that they favour the reduction of the iron, then only a small amount of $Fe^{3+}$ must be present under the conditions of these experiments. Therefore, it is unlikely that a Michaelis-Menten relationship between the concentration of the reducing agent and the enzyme activity would be found if this were the sole function of the reducing agents. Moreover, if this were the case, the maximum velocity of the enzyme should be independent of the nature of the reducing agent and this is not so.

If the reducing agents were concerned in reducing a group on the surface of the enzyme which was essential for activity and the reduction were an equilibrium reaction requiring relatively high concentrations of the reducing agent, it might be expected that there would be a Michaelis-Menten relationship between the concentration of the reducing agent and the enzyme activity. As the reducing agents are capable of reducing disulphide linkages, it would seem most likely that this was the type of group concerned. However, the
Michaelis-Menten relations indicate that the enzyme would have to be a monothiol enzyme and it is difficult to see to what this group could have been oxidised. However, if the enzyme molecules were monothiol complexes which can form dimers in solution as a result of the formation of disulphide linkages, then the activation of the enzyme by reducing agents could be concerned with the splitting of these linkages. Two molecules of the reducing agent would be required to reduce each dimer and two active centres, one on each molecule, would be exposed. The reducing agent would then be reacting with the enzyme in the ratio of one molecule of the reducing agent to one active centre of the enzyme.

However, it would be expected that if the same type of disulphide bonds were concerned that when the concentration of each reducing agent, which was capable of activating the enzyme, was increased to a sufficiently high value, the maximum enzyme activity would be independent of the nature of the reducing agent. This is not the case, so the above hypothesis must be abandoned.

Before discussing another possible interpretation of the results obtained with the reducing agents, some
complicating features of the reaction must be mentioned. The four reducing agents which were capable of activating aconitase are also capable of forming complexes with Fe$^{2+}$ and these complexes exist under the conditions used for the activation of the enzyme. It might seem as though it was the complexes which activated the enzyme, rather than the free components. As the results obtained are consistent with the idea that one molecule of the reducing agent reacts with each active centre of the enzyme, this cannot be the case. At least with cysteine and thioglycollate, the complex formed with Fe$^{2+}$ is of the type Fe$^{2+}(RS)_2$, so the results would indicate that two molecules of the reducing agent were reacting with each active centre of the enzyme. The same argument applies if the Fe$^{2+}$-reducing agent complexes were concerned in reducing the enzyme. Moreover, if the function of these complexes were simply to reduce the enzyme, it would be expected that other metals of the same transition series e.g. Co$^{2+}$, Mn$^{2+}$ etc. could replace Fe$^{2+}$.

The fact that the reducing agents, cysteine, thioglycollate and ascorbic acid, react with aconitase in the ratio of one molecule of the reducing agent to
one active centre can also be interpreted to mean that
the reaction:

enzyme + reducing agent \rightarrow enzyme-reducing agent

occurs and that the enzyme-reducing agent complex is at
least partially responsible foraconitase activity.
It cannot be wholly responsible as the enzyme shows no
activity in the presence of a reducing agent and
absence of Fe²⁺. Statistical analysis of the results
obtained with cysteine and ascorbic acid as activators
ofaconitase showed that the maximum enzyme velocities
and the hypothetical dissociation constants of the
enzyme complexes were significantly different. The
ability of cysteine to activate the enzyme was about
twice as great as that of ascorbic acid, although the
affinity of the enzyme for ascorbic acid was greater
than it was for cysteine. With thioglycollate, the
maximum enzyme velocity was found to be intermediate
between the velocities obtained with cysteine and
ascorbic acid, whilst the dissociation constant of the
enzyme-thioglycollate complex was greater than either
the enzyme-cysteine or the enzyme-ascorbic acid complexes.
This suggests that different forms ofaconitase, each
with distinct properties, are catalytically active.

The results with glutathiones somewhat complicate
the matter. There is no direct relationship between
the enzyme activity and the concentration of glutathione.
Below a certain concentration, its ability to activate
the enzyme is sharply reduced. It is interesting to
compare this result with those obtained by Kubowitz
(1935) on the reaction between \( \text{Fe}^{2+} \) and glutathione,
although the relationship between the two results is not
clear. He found that it was possible to form a CO
complex of ferrous glutathione only in the presence of
high concentrations of glutathione and concluded that
this was due to the high dissociability of the ferrous
glutathione complex. With a \( \text{Fe}^{2+} \) concentration of
\( 2 \times 10^{-3} \text{ M} \), he found that the concentration of glutathione
required to form the CO complex of ferrous glutathione
was \( 10^{-1} \text{ M} \). On this basis, a \( \text{Fe}^{2+} \) concentration of
\( 5 \times 10^{-4} \text{ M} \) would require a glutathione concentration of
\( 2.5 \times 10^{-2} \text{ M} \) which is the concentration found to be
required for the maximum activation of aconitase.

It has been established that \( \text{Fe}^{2+} \) is a specific
activator of aconitase and forms a dissociable complex
with the enzyme, also that this metal alone is not
sufficient to bring about maximum activation of the enzyme. Reducing agents are also required, in fact, the degree of activation of the enzyme by \( \text{Fe}^{2+} \) is dependent on the nature of the reducing agent present. It has also been established that cysteine, thioglycollate and ascorbic acid form dissociable complexes with the enzyme. The dissociation constant of each complex differs and bears no relationship to the maximum velocity of the enzyme. From these facts, it seems as though the activation of aconitase by \( \text{Fe}^{2+} \) and a reducing agent can best be explained by considering the active form of the enzyme to be a \( \text{Fe}^{2+} \)-enzyme-reducing agent complex. The results with glutathione do not completely fit in with this hypothesis. Glutathione does activate the enzyme, but if a \( \text{Fe}^{2+} \)-enzyme-glutathione complex is required for activity, the glutathione does not dissociate in the same fashion as do the other reducing agents. Therefore, at present the mechanism of the activation of aconitase by glutathione must be regarded as being distinct from that with the other reducing agents.

It may be only fortuitous that all the organic compounds found to activate aconitase are reducing agents. Other types of organic compound have not been
it is tempting to suggest that the remaining two free valencies of the $Fe^{2+}$ are concerned in substrate linkage. If this were the case, it is not so difficult to reconcile the fact that compounds of such different structure as cysteine and ascorbic acid can function as activators of aconitase. On the other hand, if $Fe^{2+}$ alone were concerned with linking the substrate to the enzyme, there would be, in effect, competition between the activators of the enzyme and the substrates.

Although further information has been obtained concerning the relationship between $Fe^{2+}$ and the activators and the aconitase activity, it is clear that the problem of the mode of action of aconitase is far from being solved. More light may be thrown on the problem by determination of the Michaelis constants and maximum velocities of aconitase for its three substrates in the presence of different activators, also by determination of whether or not $Fe^{2+}$ can form a mixed complex with each of the activators and the substrates of the enzyme.

**SUMMARY**

1. It was shown that the final purified aconitase preparation possessed little activity. It was not
activated by cysteine alone; the addition of Fe$^{2+}$ increased the activity 15-fold, whilst the addition of Fe$^{2+}$ and cysteine gave a 70-fold increase in activity.

2. The optimum conditions required for maximum activation and maximum activity of aconitase were determined.

3. Aconitase was not activated by Mn$^{2+}$ and Cu$^{2+}$ was inhibitory.

4. A Michaelis-Menten relationship was shown to exist between the concentration of Fe$^{2+}$ and the enzyme activity. It was concluded that a Fe$^{2+}$-enzyme complex was partly responsible for aconitase activity.

5. A Michaelis-Menten relationship was also found to exist between the enzyme activity and the concentration of the reducing agents cysteine, thioglycollate and ascorbic acid. It was concluded that these compounds form an enzyme-reducing agent complex which is partly responsible for enzyme activity. Only higher concentrations of glutathione appreciably activated aconitase; there was no Michaelis-Menten relationship between the concentration of glutathione and the enzyme activity.
6. The conclusion was drawn that the active form of ascomitate is either an enzyme-$Fe^{2+}$-reducing agent or an enzyme-$Fe^{2+}$-activator complex.
CHAPTER III

THE INFLUENCE of BUFFERS on the

pH OPTIMUM of ACONITASE
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THE INFLUENCE OF BUFFERS ON THE pH OPTIMUM OF ACONITASE

INTRODUCTION

Jacobsohn (1941) found that the pH optimum of aconitase in the presence of phosphate buffer was in the neutral region. A more extensive study of the influence of pH on aconitase activity was made by Johnson (1939). He claimed that the pH-activity curve showed a sharp maximum at pH 7.4, but this value must be regarded as approximate. The pH values chosen for testing the aconitase activity were widely spaced and no mention was made of the buffers used to cover the extensive pH range.

As this was the only information concerning the effect of pH on aconitase activity and was gained from studies with crude aconitase preparations, a more detailed investigation was made of the effect of pH
and the type of buffer on the enzyme activity.

EXPERIMENTAL

Determination of aconitase activity

Aconitase was activated with Fe$^{2+}$ and cysteine by the method previously described (page 19). The enzyme solution contained 60 µg. of the final aconitase preparation/ml. in the presence of $5 \times 10^{-4}$ M Fe$^{2+}$ and $10^{-2}$ M cysteine. The veronal acetate buffer was prepared according to Michaelis (1931), whilst the other buffers were 0.1 M, 0.15 M. Samples of the enzyme solution were added to media containing 2.5 ml. of the appropriate buffer, 0.5 ml. of cis-aconitic acid or isocitric acid (final concentration $4 \times 10^{-3}$ M) and 1.85 ml. of water. The reaction was stopped after 15 min. at 30$^\circ$C by the addition of 0.5 ml. of 50% (w/v) trichloroacetic acid when citric acid was determined, or 0.5 ml. of 10% (w/v) metaphosphoric acid when cis-aconitic acid was estimated.

Determination of citric and cis-aconitic acids

Citric acid was estimated by the method previously described (page 18), whilst cis-aconitic acid was estimated by the method of Dickman (1952).
Reagents

N-Ethyl morpholine was kindly supplied by Prof. A. Albert. Veronal, glycerophosphor nic acid, glycyl-glycine, boric and metaphosphoric acid were B.D.H. Laboratory Reagents. The other reagents were A.R.

Results

During the purification of aconitase, the various fractions obtained were tested for aconitase activity in a system which did not include a buffer and the pH chosen for the test was an arbitrary value. As it is preferable to study enzymic reactions in a system in which the pH is stabilized by the presence of a buffer, the enzyme activity was determined firstly in the presence of different buffers at pH 7.4.

Aconitase activity in the presence of different buffers at pH 7.4

It was seen in Chapter I that the inactivating effect of various buffers could be eliminated if the enzyme were added to the buffer-substrate mixture. These activity tests were carried out in that fashion. Fig. 12 shows the effect of six different buffers at pH 7.4 on the activity of aconitase as compared to the activity when the reactions were carried out in the absence
Fig 12. The activity of aconitase in the presence of different buffers at pH 7.4. Enzyme activity determined as described in the text. Temperature 30°C, buffer 0.05 M.

Unshaded bars = cis-aconitic acid → citric acid - (1)
Shaded bars = isocitric acid → citric acid - (2)
Figures indicate the ratio of reaction (1) to reaction (2).
of buffer. For comparative purposes the reactions
isocitric acid → citric acid and d-isaconitic acid
→ citric acid were studied. It is clear that the
composition of the buffer has a profound influence on these
reactions. In the reaction d-isaconitic acid → citric
acid, the enzyme activity in glycyl-glycine is decreased
as compared to the activity in the absence of buffer;
veronal acetate and N-ethyl morpholine have no effect,
whilst borate, phosphate and glycero-phosphate increase
the activity. In the reaction isocitric acid →
citric acid, the activity is approximately the same in
phosphate and glycero-phosphate, and in the absence of
buffer, whereas the activity is lower in borate, glycyl-
glycine, N-ethyl morpholine and veronal acetate. The
consequence is that the ratio of the rates of the two
reactions varies according to the buffer used.

There seemed to be two possible explanations for
the above results; that the various buffers have
different abilities to activate and inactivate the two
reactions, or that the pH optimum or optima of the
reactions are influenced by the ions present in solution.
As the latter explanation seemed more likely, the pH
optimum of the enzyme in catalysing both reactions was
determined in the presence of different buffers.
Influence of buffers on the pH optimum of aconitase

It can be seen from figs. 13 & 14 that the pH optimum of aconitase is influenced by the buffer in which the reaction is carried out. The following pH optima were found for both the reactions: glycerophosphate, 7.5; phosphate, 7.7; N-ethyl morpholine, 8.1 and veronal acetate 8.6. The absolute rates of each reaction were approximately the same at the pH optimum for each buffer, so that the ratio of the rates of the reactions cis-aconitic acid $\rightarrow$ citric acid to iso-citric acid $\rightarrow$ citric acid was about 1.8 in each case. This compares with values ranging from 1.6 to 3.5 which were obtained previously with these buffers at pH 7.4.

It was not possible to determine the activity of aconitase over a wide range of pH in the absence of buffer on account of the poor buffering capacity of the aconitase substrates at higher pH values. (The approximate pH values of these acids are reported in Table 6. It was of interest to determine them as to the author's knowledge no values have been reported in the literature). However, the activity of the enzyme was determined for the reaction cis-aconitic acid $\rightarrow$ citric acid at the lower pH values. In figs. 13 & 14 it is seen that over the pH range which could be studied, the
Fig. 13. Effect of buffers on the pH optimum of aconitase. Enzyme activities determined as described in the text. Temperature 30°, buffer 0.05 M.

- x = no buffer
- 0 = phosphate
- Δ = glycerophosphate
- ● = phosphate
- ▲ = glycerophosphate

\[
\text{cis-aconitic acid} \rightarrow \text{citric acid}
\]

\[
\text{isocitric acid} \rightarrow \text{citric acid}
\]
Fig. 14: Effect of buffers on the pH optimum of aconitase. Conditions the same as those of Fig. 13.

- $\times$ = no buffer
- $\circ$ = $N$-ethyl morpholine, cis-aconitic acid $\rightarrow$ citric acid
- $\triangle$ = veronal acetate

- $\bullet$ = $N$-ethyl morpholine, isocitric acid $\rightarrow$ citric acid
- $\triangle$ = veronal acetate
TABLE 6

PK Values of Citric, cis-Aconitic and isoCitric Acids

(0.04 M solutions of the acids were titrated with 0.073 M NaOH at 20°. Stirring was by means of a rapid stream of N₂. isoCitric acid was the DL-isomer. The values in brackets are those obtained for citric acid by Hastings & Van Slyke (1922) at 20°).

<table>
<thead>
<tr>
<th>Acid</th>
<th>PK₁</th>
<th>PK₂</th>
<th>PK₃</th>
</tr>
</thead>
<tbody>
<tr>
<td>Citric</td>
<td>3.2</td>
<td>4.7</td>
<td>5.6</td>
</tr>
<tr>
<td></td>
<td>(3.1)</td>
<td>(4.75)</td>
<td>(5.4)</td>
</tr>
<tr>
<td>cis-Aconitic</td>
<td>2.1</td>
<td>4.1</td>
<td>6.2</td>
</tr>
<tr>
<td>isoCitric</td>
<td>3.1</td>
<td>4.4</td>
<td>5.2</td>
</tr>
</tbody>
</table>
enzyme had not reached its maximum activity. If the line obtained is produced to the level of the optimal enzyme activity in the other buffers, it can be estimated that the pH optimum in the absence of buffer is about pH 8.0.

As the pH optimum of the reactions cis-aconitic acid \(\rightarrow\) citric acid and isocitric acid \(\rightarrow\) citric acid was the same in the presence of a particular buffer and altered to the same extent in different buffers, it was of interest to determine if this were also the case for the reaction isocitric acid \(\rightarrow\) cis-aconitic acid. The pH-activity curves obtained with phosphate and veronal acetate buffers, as shown in fig. 15, indicate that this is indeed the case.

**Inactivation of aconitase by buffers**

Fig. 16 illustrates the rapid inactivation of aconitase that occurs when the enzyme is incubated in the absence of substrate for various periods of time with different buffers. The pH of each buffer was such that the aconitase activity was maximal. The rate of inactivation differs for each buffer and appears to be a function of the pH value.
Fig. 15. Effect of buffers on the pH optimum of aconitase. Conditions the same as those described in Fig. 13 except that the reaction was stopped by the addition of 0.5 ml. of 10% (w/v) metaphosphoric acid.

* = phosphate
* = isocitric acid → cis-aconitic acid
x = veronal acetate
Fig. 16. Inactivation of aconitase by buffers in the absence of substrate. Aconitase activated with Fe^{2+} and cysteine was incubated at 30° for various periods of time in the presence of 0.05 M buffer. citro-
acetic acid was added and the reaction stopped after 15 min. Solutions were analysed for citric acid.

x = glycerophosphate (pH 7.5)
o = phosphate (pH 7.7)
• = N-ethyl morpholine (pH 8.1)
▲ = veronal acetate (pH 8.6)
DISCUSSION

These results explain not only the different activities which were found in various buffers at pH 7.4, but also the apparent activation ofaconitase by phosphate buffer at pH 7.4, as reported in Chapter I. The increases in enzyme activity are not due to the activation of the enzyme, but to the fact that the pH value chosen was closer to the optimum value in some buffers than it was in others. The alteration of the optimum pH of an enzyme is not a new phenomenon, but it is unusual to find that it is shifted to such an extent. Massey (1953) has reported similar effects of ions on the pH optimum of fumarase.

Michaelis & Davidsohn (1911) pointed out that the pH-activity curve of sucrasease could be explained by assuming that the enzyme was an ampholyte so that the two halves of the pH-activity curve corresponded to the dissociation of the acidic and basic groupings on the surface of the enzyme. They suggested that the enzyme showed maximum activity when it existed in the isionic form so that when the pH was altered in either direction, the amount of enzyme available for combination with the substrate was reduced. Thus, the enzymic activity was
decreased. Kuhn (1923) showed that the fact underlying the pH-activity curve of saccharase was the variation with pH of the rate of breakdown of the enzyme-substrate complex into enzyme and products. The theories of Michaelis were therefore rejected.

There is no reason to suppose that the influence of pH on all enzymes is due to the same factors. Before any attempt is made to explain the reasons for a particular pH-activity curve, it is necessary to determine both the pH-Michaelis constant and pH-maximum velocity curves (see Dixon, 1953). In the final analysis, it is also necessary to consider the effects of pH on the stability of the enzyme, the ionisation of the substrate and the state of aggregation of the enzyme molecules. As these factors have not been investigated, no attempt will be made to explain the pH-activity curves obtained with aconitase, or the effect of ions on the position of the pH optimum. However, some points, as far as the present results are concerned, might be mentioned.

It is interesting that the buffers which depend on anions for their buffering capacity shift the pH optimum to the acid side of the pH optimum obtained in the absence
of buffer. Cationic buffers, on the other hand, shift the pH optimum to the alkaline side. It is also noteworthy that the pH optimum in each buffer lies at the end of the alkaline range of the buffer, so that it was not possible to follow the alkaline branch of the pH-activity curve to an appreciable extent.

It is significant that the pH optimum of aconitase in catalysing the three reactions \textit{isocitric acid} $\rightarrow$ citric acid, \textit{isocitric acid} $\rightarrow$ \textit{cis-aconitic acid} and \textit{cis-aconitic acid} $\rightarrow$ citric acid is the same. This would seem to indicate that not only was the same enzyme responsible for catalysing these reactions, but also that the properties of the active centre(s) were similar. It might be supposed from these results that the same catalytic centre(s) was concerned in carrying out all the reactions associated with aconitase activity.

The inactivation of aconitase by buffers in the absence of substrate is likely to be a non-ionic reaction on account of the relatively slow rate of the inactivation. In the case of the inactivation by phosphate, it could be due to the interaction of the free Fe$^{2+}$ with the phosphate so as to upset the equilibrium between the free enzyme, free Fe$^{2+}$ and the Fe$^{2+}$-enzyme complex. The attainment of the new equilibrium
would be a slow process. The ionic reaction between phosphate and the free Fe\(^{2+}\) would conceivably be rapid, but the rate at which the Fe\(^{2+}\) was removed from the enzyme would be slow, as this reaction is the reverse of the activation process. However, this theory would not hold in the case of N-ethyl morpholine as this buffer does not form co-ordination compounds with Fe\(^{2+}\). As the rate of inactivation increases with a rise in the pH, it seems more likely that the inactivation of the enzyme is not concerned specifically with Fe\(^{2+}\), but with a number of groups on the enzyme surface.

The ability of the substrates of the enzyme to prevent the inactivation which occurs in these buffers must be due to the fact that the enzyme has a greater affinity for the substrates than it has for the buffer ions. Nevertheless, according to the theory of enzyme action postulated by Michaelis & Menten (1913), there is always a small amount of enzyme which is not combined with substrate. Therefore, it is likely that the substrate protects the enzyme to a large extent, but not completely.

SUMMARY

1. It has been shown that the pH optimum of aconitase
is influenced by the ions present in solution.

2. The pH optimum of aconitase in catalysing the reactions isocitric acid $\rightarrow$ citric acid, isocitric acid $\rightarrow$ cis-aconitic acid and cis-aconitic acid $\rightarrow$ citric acid is the same in the presence of a particular buffer and altered to the same extent in different buffers.

3. The inactivation of aconitase by buffers in the absence of substrate has been shown.

4. The significance of these findings has been discussed.